Fetal ethanol exposure increases ethanol intake by making it smell and taste better

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Human epidemiologic studies reveal that fetal ethanol exposure is highly predictive of adolescent ethanol avidity and abuse. Little is known about how fetal exposure produces these effects. It is hypothesized that fetal ethanol exposure results in stimulus-induced chemosensory plasticity. Here, we asked whether gestational ethanol exposure increases postnatal ethanol avidity in rats by altering its taste and odor. Experimental rats were exposed to ethanol in utero via the dam's diet, whereas control rats were either pair-fed an iso-caloric diet or given food ad libitum. We found that fetal ethanol exposure increased the taste-mediated acceptability of both ethanol and quinine hydrochloride (bitter), but not sucrose (sweet). Importantly, a significant proportion of the increased ethanol acceptability could be attributed directly to the attenuated aversion to ethanol's quinine-like taste quality. Fetal ethanol exposure also enhanced ethanol intake and the behavioral response to ethanol odor. Notably, the elevated intake of ethanol was also causally linked to the enhanced odor response. Our results demonstrate that fetal exposure specifically increases ethanol avidity by, in part, making it taste and smell better. More generally, they establish an epigenetic chemosensory mechanism by which maternal patterns of drug use can be transferred to offspring. Given that many licit (e.g., tobacco products) and illicit (e.g., marijuana) drugs have noteworthy chemosensory components, our findings have broad implications for the relationship between maternal patterns of drug use, child development, and postnatal vulnerability.

chemosensory plasticity | ethanol acceptability | olfaction | bitter | gustation

Fetal ethanol exposure can have significant negative consequences for the developing human fetus. Fetal alcohol spectrum disorder describes a continuum of sequelae that can range from craniofacial malformations and mental retardation to behavioral changes such as hyperactivity and learning and memory deficits (1). There are also subtler, but nevertheless significant, clinical effects of fetal ethanol exposure. For instance, human epidemiologic studies (2, 3) report that (*i*) fetal ethanol exposure increases the risk of adolescent ethanol abuse, (*ii*) gestational exposure is the best predictor of adolescent abuse, and (*iii*) the earlier the age of secondary postnatal ethanol exposure, the greater the chance of long-term alcoholism. Little is known about how fetal exposure produces these changes in ethanol avidity.

Numerous studies have examined how ethanol consumption during pregnancy alters the perception, preference, and consumption of ethanol by the mother's offspring. Animal studies (4, 5) have established that (*i*) ingested alcohol diffuses into amniotic fluid, (*ii*) fetuses ingest amniotic fluid and sense intrauterine ethanol, and (*iii*) the fetus can acquire information about ethanol-related sensory cues and display a memory of this prenatal experience. Likewise, human studies indicate that the fetus can detect and remember prenatal experience with a variety of odorous substances present in the pregnant mother's diet (6, 7), including ethanol (8). Together, these studies show that fetal ethanol exposure changes the responsiveness of offspring to specific chemosensory attributes of ethanol. Nonetheless, the question of how fetal experiences with ethanol produce these chemosensory changes and whether they directly influence ethanol intake remains open.

Dietary manipulations during gestation can permanently alter gustatory function (9). Moreover, postnatal dietary exposure to sweet and bitter taste stimuli can increase their acceptability (10–13). In Exp. 1, we asked whether fetal ethanol exposure promotes ethanol intake by making it taste "better." Previous rat studies have shown that binary mixtures of sucrose and quinine hydrochloride (QHCl) create an oral sensation (14, 15) and neurophysiologic response (14) similar to that of ethanol. These studies indicate that the sweet taste of sucrose and bitter taste of QHCl constitute salient components of the oral sensation generated by ethanol. We hypothesized, therefore, that if fetal ethanol exposure enhances ethanol intake, it might do so by intensifying its sweetness and/or attenuating its bitterness. To test this hypothesis, we determined whether fetal ethanol exposure increases the orosensory acceptability of ethanol and, if so, whether the increase is directly mediated by a reduction in its quinine-like taste quality or an enhancement of its sucrose-like taste quality.

In Exp. 2, we asked whether fetal ethanol exposure further increases ethanol acceptability by making it smell better. Previously, it has been reported that fetal exposure to ethanol "tuned" both the peripheral neural and unconditioned behavioral olfactory response to ethanol odor in infant rats [postnatal day (P) 15] (16). A parallel study showed that fetal ethanol exposure also enhanced ethanol intake (17). This constellation of prenatal exposure effects persisted into the "at-risk" age of adolescence (i.e., P30–P42) (4, 5, 18, 19). Despite this earlier work, however, there is still no direct evidence that fetal ethanol exposure increases ethanol intake by enhancing its olfactory attributes. Exp. 2 provides this direct evidence.

Results

Exp. 1: Does Fetal Ethanol Exposure Make Ethanol Taste Better by Intensifying Its Sweetness or Reducing Its Bitterness? We tested both adolescent (P30) and adult (P90) rats. P30 was chosen because it is the earliest age readily amenable to lickometer testing and because fetal ethanol exposure has consistently been shown to enhance ethanol intake in adolescent rats (4, 5). We tested P90 rats to determine whether any effect of fetal ethanol exposure on taste-guided behavioral responses persists into adulthood.

Acceptability of Ethanol, QHCl, and Sucrose to P30 Rats. Fig. 1 *A* and *B* illustrates the licking rates of P30 rats (expressed as a ratio of licks to the chemical stimulus relative to water) in the 3 maternal

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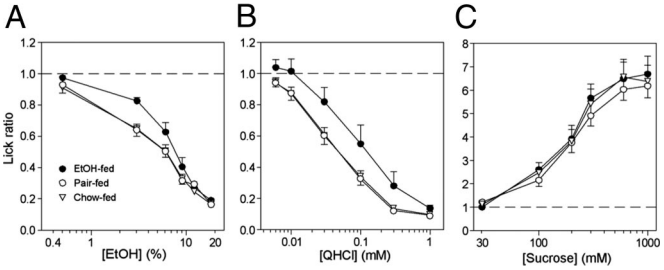

Fig. 1. Fetal exposure increases the acceptability of ethanol (*A*) and QHCl (*B*), but not sucrose (*C*). Acceptability is expressed as a lick ratio (mean ± SEM). A ratio of 1.0 (dashed line) occurs when licks to the tastant equal licks to water. Ratios <1.0 indicate the solution inhibited licking, whereas those >1.0 indicate it stimulated licking. Note: the scale on the *y* axis of *C* differs from that of *A* and *B*.

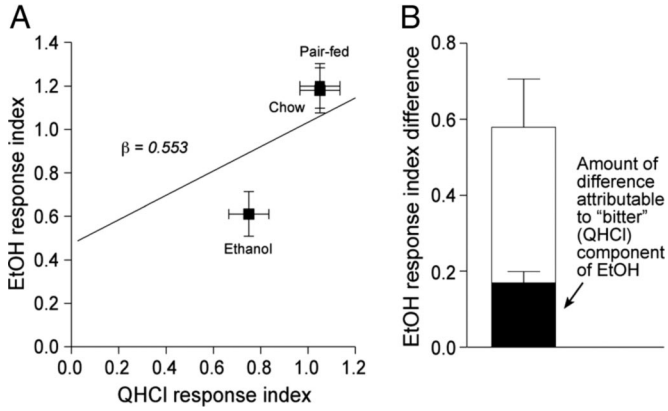

Fig. 2. The attenuated licking response to 3% and 6% ethanol can be attributed to the increased acceptability of its bitter-like taste. (*A*) The estimated slope of the predictive relationship between the animal's orosensory response index to QHCl (a surrogate for ethanol's bitter taste) and the same animal's response index to ethanol. The slope is shown relative to the mean (± 2D standard errors) location of each maternal treatment group. (*B*) The full height of the column is the net difference in the ethanol response index between ethanol and chow animals. The solid black portion represents the contribution to the net ethanol effect that can be ascribed to fetal ethanol's effect on the response to ethanol's bitter taste. The data are the means ± SEM.

treatment groups across a range of ethanol and QHCl concentrations. Although the rats in all treatment groups exhibited a concentration-dependent decrease in licking rate for both stimuli, there were marked maternal treatment effects. Whereas the curves for the progeny of pair-fed and chow-fed dams were indistinguishable from each other, those from the progeny of ethanol-fed rats had higher lick ratios for 3% and 6% EtOH (Fig. 1*A*) and for the range of 0.006 to 0.3 mM QHCl (Fig. 1*B*). Further, compared with controls, 0.006 and 0.01 mM QHCl yielded lick ratios equivalent to water (i.e., = 1), suggesting that these concentrations were either at or below threshold detection for the ethanol-fed animals. It follows that the rightward shift of the concentration-response curve for QHCl may represent a decrease in sensitivity. Alternatively, the shift in the response may reflect a change in hedonic response of the ethanol-fed animals. At present, we cannot distinguish between these possibilities.

For ethanol (Fig. 1*A*), the dynamic range of the prenatal effect is between 3% and 6% ethanol. Notably, these same concentrations of ethanol represent a stimulus range of enhanced voluntary ethanol intake demonstrated by others (20, 21) in adolescent rats.

Fig. 1*C* shows licking responses of P30 rats to a range of sucrose concentrations. Rats in each group displayed largely overlapping concentration-dependent increases in licking ratios, indicating that prenatal exposure did not alter the responses to sucrose.

In the description of the data above, the responses to the 6 concentrations of each tastant represent a multivariate set of correlated variables in a repeated measures experiment. To make valid estimates regarding the effect of prenatal treatment on the overall responsiveness to each tastant, we performed separate randomized-blocks ANOVAs for each tastant that accounted for the animal's multivariate responses for each chemical tested. For ethanol and QHCl, there was a significant effect of prenatal treatment [F (2,25) = 8.49 and 5.10, $P < 0.002$ and 0.015, respectively]. There was no sex effect [F (1,25) = 0.06 and 0.53, respectively, all $P > 0.4$] or sex × maternal treatment interaction [F (1,25) = 0.54 and 1.49, respectively, in both cases $P > 0.2$].

For sucrose, there was no evidence of a maternal treatment effect on lick responsiveness [F(2,25) = 0.20, $P > 0.80$].

Taken together, the data demonstrate that fetal ethanol exposure significantly attenuated the aversive response to ethanol and QHCl, but failed to increase the appetitive response to sucrose in P30 rats.

The Contribution of the Attenuated Aversion to Bitter (QHCl) on the Response to Ethanol. Humans perceive ethanol as a combination of both sweet and bitter tastes (22, 23). Likewise, conditioned-generalization tests indicate that rats perceive ethanol as having a sweet (sucrose-like) and bitter (QHCl-like) taste (14, 15). Thus, we asked whether the attenuated aversion to bitter (represented by the response to QHCl) accounts for part of the increased acceptance of 3% and 6% ethanol.

We first tested for a predictive relationship between overall responsiveness to QHCl and that to 3% and 6% ethanol. However, the multivariate nature of the data for each tastant did not permit a direct assessment of such a relationship. Therefore, we took an approach that is common in the field of psychology, namely, to create an "index" that incorporates the animal's behavioral responses into a single principal measure. Following a previously established approach (16, 18, 19, 24) we generated a composite behavioral index that incorporates the rats' licking responses to all QHCl concentrations (henceforth, the QHCl orosensory response index) (see *Materials and Methods*). Further, we generated a reduced composite ethanol orosensory response index that integrated the rats' licking responses to the 2 relevant concentrations of ethanol (namely, 3% and 6%). To estimate the ability of the QHCl response index (a surrogate for ethanol's bitter taste) to predict changes in the ethanol response index, we performed an analysis of covariance (ANCOVA) (16). In this analysis, the slope, β, was the coefficient of X in the regression of Y (the ethanol responses of the 3 maternal treatment groups) on X (the QHCl behavioral responses of the same animals). The results demonstrated a significant slope [F (1,29) = 4.95; $P < 0.035$], where the estimate of $\beta = 0.553$ (Fig. 2*A*). Thus, a linear predictive relationship exists between the effect of fetal ethanol exposure on the responsiveness to bitter (as assessed with QHCl) and that to 3% and 6% ethanol.

To determine whether the effect of in utero ethanol exposure on ethanol acceptance was caused (at least in part) by a change in responsiveness to ethanol's bitter taste quality, we could not rely on the predictive relationship illustrated in Fig. 2*A*. Instead, we had to incorporate into the analysis the strength of the maternal treatment effect (16) on the response to QHCl (i.e., the difference in the QHCl orosensory response index between the ethanol- and chow-fed rats). To this end, we multiplied the slope (β) of the predictive relationship in Fig. 2*A* times the difference in QHCl orosensory response index between ethanol- and chow-fed rats. We used the chow-fed (as opposed to pair-fed)

rats as controls because (*i*) pair-feeding is a form of perturbation and (*ii*) there is no evidence of a differential effect between the pair-fed and chow-fed rats on licking for either of the 2 chemical stimuli (Fig. 1*A* and *B*) or on the predictive relationship between the composite QHCl and ethanol indexes in the same animals (Fig. 2*A*). Using this approach, we determined that a significant proportion of the net differential response to 3–6% ethanol was attributable to an effect of prenatal ethanol exposure on responsiveness to bitter taste [$t(22) = 5.63$, $P < 0.00002$].

Fig. 2*B* (empty column) shows that the estimated effect of fetal ethanol exposure on the ethanol response index of ethanol-fed versus chow-fed rats is 0.578 ± 0.128 (mean ± SEM). This value represents the net total effect of prenatal ethanol exposure on ethanol orosensory acceptability. Fig. 2*B* (filled column) shows the product value from the analysis above [i.e., the difference in the mean QHCl response indices between chow-fed ($\mu_{X1} = 1.102$) and ethanol-fed ($\mu_{X2} = 0.795$) rats times the slope ($\beta = 0.553$) of the predictive relationship, namely, 0.169]. This value represents the partial effect of prenatal exposure on the net ethanol orosensory acceptability that is attributable to the reduced aversion to bitter. The ratio of these 2 estimates demonstrates that the reduced aversion to bitterness (i.e., QHCl) accounts for an estimated 29.2% of the fetal exposure effect on the ethanol response.

Acceptability of Ethanol, QHCl, and Sucrose to P90 Rats. For the 3 stimuli, there was no evidence of a maternal treatment effect in the P90 rats, showing that the effects observed in adolescent rats do not persist into adulthood (all ANOVAs, $P > 0.35$). Thus, we did not evaluate the relationship between the QHCl and ethanol responses.

Exp. 2: Does the Behavioral Response to Ethanol Odor Contribute Directly to Enhanced Ethanol Intake? Given the role of odor in flavor perception we asked whether fetal ethanol exposure increased ethanol acceptability by altering the response to its odor. To this end, we examined both unconditioned sniffing responses to ethanol odor and short-term ethanol intake in the same animal. As described, the monitoring of reflexive sniffing provides a means for assessing the attentiveness/responsivity to odorant stimuli (24). We again used offspring of ethanol-, pair-, and chow-fed dams. We selected P15 rats because previous observations have revealed a parallel relationship between gestational exposure, olfactory function, and ethanol avidity at this age (16, 17).

Reflexive Sniffing Response to Ethanol Odor. Subjects were first tested for stimulus-induced reflexive sniffing responsiveness to ethanol odor. Because the airflow patterns generated by sniffing are complex, they cannot be adequately described or quantified by examining a single variable (e.g., frequency) (24, 25). Responses to ethanol odor stimulation were quantified by compressing 14 descriptive response variables into 2 uncorrelated variables with principal components analysis (PCA). Further, in keeping with the logic outlined above for the orosensory data we created an index that incorporates the animal's behavioral responses across concentrations into a single primary measure (16, 18, 19, 24) (see *Materials and Methods*).

Fig. 3*A* illustrates a 2D composite sniffing index solution, based on the resultant 2 PCA factors, with the mean of each maternal treatment group representing points in behavioral response space. The difference between any 2 treatment groups reflects the degree to which their inherent behavioral responses to ethanol odor are dissimilar in the multidimensional behavioral space. As seen in Fig. 3*A*, there is a clear separation between the ethanol- and control-fed groups. Randomized-blocks multivariate ANOVA was performed with each animal's responses in 2 dimensions as the dependent variables. The results confirmed

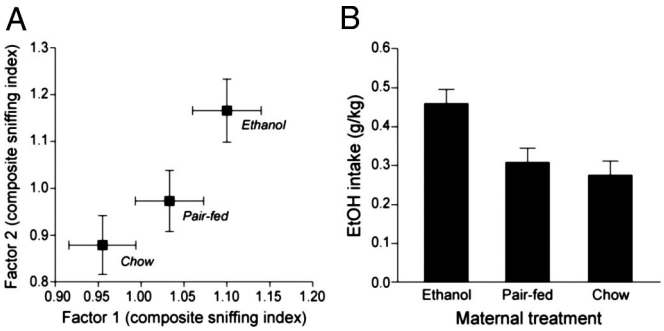

Fig. 3. Fetal ethanol exposure alters the reflexive sniffing response to ethanol odor and ethanol intake. (*A*) The mean (± 2D standard errors) composite sniffing indexes for the maternal treatment groups. (*B*) Illustration of the grams of absolute ethanol consumed per kg of body weight for the P15 rats as a function of maternal treatment. The data are the means ± SEM.

our previous observation of a significant maternal treatment effect (16) [$F(4,92) = 3.324$, $P < 0.02$]. There was no evidence of a differential sex effect [$F(2,46) = 0.314$, $P > 0.70$], or a sex × treatment interaction [$F(4,92) = 0.534$, $P > 0.50$].

Ethanol Intake. After olfactory testing, each animal was evaluated for ethanol intake. Fig. 3*B* illustrates how grams of absolute ethanol intake per kilogram body weight (EtOH g/kg) varied as a function of maternal treatment. In keeping with our previous results (17) and those of others (4), we found that fetal ethanol exposure increased postnatal ethanol intake. Randomized-blocks ANOVA revealed a significant effect of prenatal treatment on ethanol acceptance [$F(2,49) = 4.74$, $P < 0.013$]. There was no evidence of a differential sex effect [$F(1,49) = 0.1226$, $P > 0.70$] or sex × treatment interaction [$F(2,49) = 1.61$, $P > 0.20$].

The Contribution of Ethanol Odor to Ethanol Intake. Next, we assessed the extent to which the altered response of ethanol odor (stemming from fetal ethanol exposure) contributed directly to the observed increase in ethanol intake. As in Exp. 1, we first asked whether the animals' unconditioned behavioral response to ethanol odor reliably predicted ethanol intake. A priori we used each rat's composite reflexive sniffing index (derived from factor 1 of the PCA) to define its sniffing response to ethanol odor. In this respect, it has been shown that a significant proportion of the observed composite sniffing response (based on factor 1) is attributable to prenatal ethanol's effect on the neural response of the olfactory epithelium (16). Thus, for each rat tested, there was 1 measure of odor-mediated sniffing response and 1 measure of absolute ethanol intake. We again performed an ANCOVA to test $H_0{:}\beta = 0$, where β was the coefficient of X, in the regression of Y (the ethanol intake responses of the ethanol-, pair-, and chow-fed animals) on X (the odor-mediated sniffing responses of the same animals). The ANCOVA revealed a significant predictive relationship [$F(1,52) = 6.36$; $P < 0.015$] with an estimate of $\beta = 0.336$ (Fig. 4*A*).

To establish a causal association between the maternal treatment effects on olfactory function and ethanol intake, we estimated the magnitude of the effect of prenatal exposure on the olfactory behavioral response. To test the null hypothesis, $H_0{:}(\mu_{X1} - \mu_{X2})\beta = 0$, we compared the composite sniffing index of the ethanol-fed and chow-fed rats. We again used the chow-fed controls in this comparison because in addition to the rationale noted above the progeny of ethanol-fed rats ingested significantly more ethanol than either pair-fed or chow-fed controls (Tukey's posthoc tests: $P < 0.025$ and 0.009, respec-

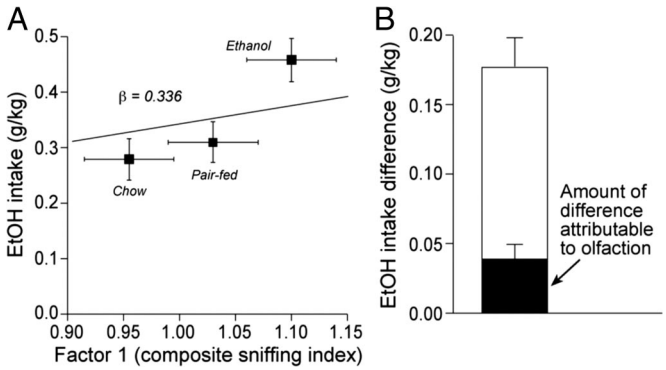

Fig. 4. Increased ethanol intake can be attributed to the enhanced behavioral response to its odor. (*A*) The estimated slope of the predictive relationship between the behavioral response to ethanol odor and ethanol intake. The slope is shown relative to the mean (± 2D standard errors) location of each maternal treatment group. (*B*) The height of the column is the net difference in absolute ethanol intake (g/kg) between ethanol and chow animals. Solid black portion is the contribution to the total effect directly attributable to the response to ethanol odor. The data are the means ± SEM.

tively), and there was no difference between pair- and chow-fed animals ($P > 0.80$; see Fig. 3*B*). This analysis established that a significant portion of the net differential ethanol intake response is directly explained by prenatal ethanol's effect on the response to ethanol odor [$t(42) = 5.04$, 2-tailed, $P < 0.0001$].

To quantify the partial contribution, we multiplied the difference between the mean chow-fed ($\mu_{X1} = 0.965$) and ethanol-fed ($\mu_{X2} = 1.087$) composite sniffing indices (Fig. 4*A*) times the slope ($\beta = 0.336$) of the predictive relationship between the sniffing response to ethanol and ethanol intake. The product, 0.0393 g/kg of absolute EtOH, provides an estimate of the olfactory partial contribution to the net effect of fetal exposure on absolute ethanol intake (Fig. 4*B*; filled column). The net total effect of maternal treatment on ethanol intake between ethanol- and chow-fed rats was 0.178 g/kg of absolute EtOH (Fig. 4*B*, empty column). The ratio of 0.0393 g/kg to 0.178 g/kg shows that 22.1% of the enhanced ethanol intake effect can be attributed directly to an enhanced behavioral response to ethanol.

Discussion

The chemosensory attributes of ethanol (i.e., smell, taste and somatosensory) are thought to be important determinants of ethanol acceptance (26). In this respect, 2 hypotheses have been proposed to explain the increased postnatal ethanol acceptance as a consequence of prior fetal exposure (5). First, during fetal exposure, the animal associates the drug's reinforcing properties with its chemosensory attributes. Second, the mere exposure to ethanol enhances the animal's "liking" for ethanol, leading to increased intake. Regarding the first hypothesis, there is evidence that prenatal exposure enhances postnatal acceptance through a conditioned response acquired by the association of the chemosensory and postabsorptive properties of ethanol (5, 20). Nonetheless, this observation does not exclude the potential contribution of an increased avidity for the chemosensory properties of ethanol. To be sure, prenatal and postnatal exposure to salient stimuli can alter the responsiveness of the taste (9, 27, 28) and olfactory (16, 29–31) systems.

Given the prominent role of oral chemosensation in controlling ethanol intake (26), we hypothesized that the effects of fetal ethanol exposure on ethanol intake are mediated, at least in part, by changes in the perceived intensity of the different quality components of ethanol. Studies indicate that the oral sensations produced by ethanol consist of both appetitive (sweet) and aversive (bitter taste and oral irritation) components (14, 15, 26, 32). Exp. 1 demonstrated that fetal ethanol exposure significantly increased the taste-mediated acceptability of both ethanol and QHCl (bitter), but not sucrose (sweet), in adolescent rats. Although previous investigators have reported that postnatal exposure to bitter taste stimuli can increase the acceptability of both the same and novel bitter taste stimuli to rodents (10, 11, 33), our work establishes that prenatal exposure to a bitter taste stimulus can produce similar effects. More importantly, we causally linked the attenuated aversion to ethanol's quinine-like taste attributes to an increased avidity for 3% and 6% ethanol. A likely consequence of diminishing the bitter component of ethanol would be a corresponding increase in its sweet component, rendering it more palatable, which could have occurred through a shift in the asymmetric suppression of the different taste qualities (34, 35). In other words, because the perceived intensity of the component taste qualities of a stimulus are known to mutually (yet unevenly) suppress each other, a reduction in the perceived bitterness of ethanol could enhance its sweet quality.

Exp. 1 also demonstrated that the increased acceptability of ethanol and QHCl to P30 animals was absent in P90 adult animals. This finding agrees with previous observations that the olfactory and ethanol intake effects of fetal exposure, while present in infant (16, 17) and adolescent rats (4, 5, 18, 19), ameliorate by adulthood (16, 17). Even so, the conjunction of these data with the clinical literature (2, 3) point to the importance of adolescence as a critical transition period for perpetuating mechanisms that contribute to the development of alcoholism.

We previously found that (*i*) infant rats exposed to ethanol throughout gestation displayed a tuned neural and behavioral olfactory response that was specific to ethanol odor (16) and (*ii*) that this observation was paralleled by enhanced ethanol intake (17). These observations raised the clinically relevant possibility that the effects of fetal ethanol exposure on olfactory function may be an important contributor to an enhanced postnatal avidity for the drug. The results of Exp. 2 critically extend this work by unambiguously demonstrating that (*i*) the effect of prenatal exposure on the response to ethanol odor significantly predicts the prenatal effect on enhanced ethanol intake in P15 rats, and (*ii*) more importantly, there is a significant causal relationship between these effects. This causal relationship may stem from either a decreased response to ethanol odor (i.e., a decreased sensitivity resulting in a reduced aversion) or an enhanced preference. In this regard, the plethysmograph data did not assign either a positive or negative valence to the observed change in sniffing responses. Nevertheless, several lines of evidence are consistent with the interpretation that fetal ethanol exposure enhanced the preference for ethanol odor: (*i*) fetal exposure results in a tuned neural and behavioral response to ethanol odor with no altered behavioral response to a nonfetally exposed odorant (16), (*ii*) other forms of chronic prenatal (29) and postnatal (36) odor experience have been shown to enhance behavioral sensitivity and preference for the exposure odorant, and (*iii*) prenatal ethanol exposure results in a preference for its odor (37). It should be noted that the parsimonious explanation of an enhanced valence effect on ethanol odor would be true even in the face of potential untoward consequences of fetal exposure on olfactory development (38). The olfactory system is functionally plastic and there is evidence demonstrating the system's ability to maintain normal or near-normal detection sensitivity (39) and odorant quality discrimination (40) after vast damage to the epithelium or bulb.

The present experiments indicate that fetal exposure enhances ethanol intake, in part, by making it taste and smell better. Not surprisingly, the effect of fetal exposure on taste and smell did not account for the entire net differential acceptability between ethanol-treated and control animals. Thus, there are other

neural pathways involved in ethanol acceptance affected by fetal exposure. With specific regard to the chemical senses, the flavor components of ethanol also include the perception of oral irritation conveyed through the trigeminal system. Fetal ethanol exposure is known to reduce the number of trigeminal neurons in the brainstem nuclei (41). This observation, in turn, could be expected to reduce the number of stimulus channels important to the perception of oral irritation. Within the context of the present set of experiments, studies examining the consequences of gestational exposure on the perception of oral irritation have not been the subject of published investigation. Nonetheless, studies demonstrate the importance of a reduction in stimulus specific channels in ethanol acceptance. Ethanol's trigeminal stimulant effects are partially mediated through the TRPV1 receptor (42). Genetic deletion of the TRPV1 receptor in mice decreases the aversive orosensory responses to ethanol (43). Likewise, repeated transient exposure to ethanol in humans reduces the perception of its oral irritancy (44). Thus, given that maternal ethanol readily provides prolonged fetal exposure, it is likely that it will also reduce oral irritation in response to ethanol, thereby further enhancing ethanol acceptability.

Conclusion

The field of environmental epigenetics has revealed that environmental contaminants can alter patterns of gene expression in the developing fetus, causing changes in nervous system development and function, homeostatically-controlled processes, and the incidence of cancer. Here, we describe an epigenetic mechanism by which maternal patterns of drug use can be transferred to offspring: fetal ethanol exposure altered development of the smell and taste systems so that the normally aversive odor and flavor of ethanol became more acceptable, thereby enhancing intake. Given that many licit (e.g., tobacco products) and illicit (e.g., marijuana) drugs have chemosensory components, our findings have broad implications for the relationship between maternal patterns of drug use, child development, and postnatal vulnerability.

Materials and Methods

Experimental Treatment of Pregnant Dams. On gestational day (G) 5, Long–Evans female rats (Harlan-Sprague–Dawley) were divided into blocks of 3 weight-matched dams and randomly assigned to either the ethanol, pair-fed, or chow group. Ethanol dams were fed an ad libitum liquid diet (L10251; Research Diets) that provided 35% of daily calories from ethanol during G11–G20, after weaning onto the diet from G6 to G10 (16, 17). Peak blood ethanol concentration was ≈150 mg/dl (16). This approach yields a relatively consistent exposure level that models moderate ethanol intake during the time when (*i*) olfactory neurons begin to transduce information in rat fetuses (i.e., G14) (45), and just before their developing axons have reached the olfactory bulb (46), and (*ii*) early dietary manipulations modify taste receptor cell transduction and produce permanent developmental and behavioral changes in the taste system (9).

The pair-fed dams received an iso-caloric, iso-nutritive liquid diet (L100252; Research Diets) matched to the volume consumed by their respective ethanol dam on the previous day. The chow dams had continuous access to lab chow and water.

Test Subjects. Within 24 h of birth, litters were culled to a maximum of 10 pups (approximately equal numbers of males and females) and fostered to dams fed standard chow and water. For Exp. 1, 6 blocks of 3 dams were used. One randomly-selected animal of each gender from any given litter was allocated to the P30 and 1 was allocated to the P90 ages, yielding 6 male and 6 female subjects from each maternal treatment group at each age. For Exp. 2, an additional 10 blocks of 3 dams were used. One randomly-selected animal of each gender from any given litter was used, yielding 10 male and 10 female subjects from each maternal treatment.

Assessment of Taste Response. To assess taste responsiveness to ethanol, sucrose, and QHCl, we measured lick responses during repeated 10-s trials (47, 48). We used a computer-controlled gustometer that presented individual chemical stimuli according to a predetermined schedule and recorded licking responses (Davis MS160-Mouse; DiLog Instruments). Each rat was subjected to 3 days of training in the gustometer. The training sessions for the P30 animals began on P27 and those for P90 rats began on P87.

To motivate the rat to lick from the sipper tube during training, it was water-deprived for 22.5 h in its home cage. Each training session lasted 30 min. On training day 1, the rat was permitted to drink water freely from a single stationary spout for 30 min, and then returned to its home cage where it was given 1 h of ad libitum access to water. The rat was again water-deprived for another 22.5 h. On training day 2, the rat had access to water during 10-s trials, separated by a 7.5-s intertrial interval; animals were able to initiate up to 205 trials across the 30-min test session. Each 10-s trial began after the first lick was detected. After this training session, the rat was water-deprived for another 22.5 h. On training day 3, the same previous day's procedure was used except the sipper tubes contained a very palatable concentration of saccharin (20 mM).

After sipper tube training, the rats were subjected to a single 30-min test session on each of 3 separate days, using the testing parameters described above. During a single daily session, we tested a range of concentrations either of sucrose (0.03, 0.1, 0.2, 0.3, 0.6, or 1.0 M), QHCl (0.006, 0.01, 0.03, 0.1, 0.3, or 1.0 mM), or ethanol (0.5%, 3%, 6%, 9%, 12%, or 18%). We also included water as a test stimulus in each test session. For a given tastant, the order of presentation was randomized without replacement in blocks so that every concentration of a taste stimulus and water was presented once before the initiation of a second block.

We tested each chemical stimulus on separate days, in the following order: EtOH, QHCl, and then sucrose. We interjected a recovery day between each test session, during which food and water were available ad libitum. To motivate avid licking for the sucrose solutions, the rats were food-deprived for 23 h before testing. To motivate avid licking for the ''aversive'' solutions (QHCl and ethanol), the rats were water-deprived for 23 h before testing.

We converted all licking responses to a lick ratio, which involved dividing the mean number of licks per trial for each taste stimulus concentration by the mean number of licks per trial for water alone. It was necessary to use this lick ratio so as to control for individual differences in both local lick rate and motivational state.

The responses to the 6 concentrations of each tastant represented a multivariate set of correlated response variables. Therefore, we constructed a set of univariate composite tastant response indexes (3 values for each rat) that represented each animal's overall behavioral lick response to ethanol, QHCl, and sucrose. For each tastant we performed a multivariate regression analysis with the 6 tastant-induced lick response measures as the dependent variables and maternal treatment as the independent variable. This approach provided estimates of the coefficients for each concentration of tastant in the separate regression analyses. The composite index value for each animal was the linear summation of the constant from the analysis, plus the respective lick ratio value at each of the concentrations of tastant multiplied by the appropriate estimated coefficient. These data were used in subsequent analyses.

Monitoring of Stimulus-Induced Reflexive Sniffing. Using whole-body plethysmography, we monitored changes in respiration in response to the presentation of air or odorant into a chamber through which a constant stream of airflow was delivered (16, 18, 19, 24). After a habituation period, air and ethanol odor stimuli were randomly presented in blocks of 20 trials (10 air and 10 odorant). An ascending series of 5 ethanol concentrations (expressed as the fraction of vapor saturation at 20 °C: 3.125×10^{-3}, 6.25×10^{-3}, 1.25×10^{-2}, 2.5×10^{-2}, and 5×10^{-2}, respectively) was used such that each concentration was presented for 1 block of 20 trials. For each 6-s stimulus presentation the sniffing patterns were analyzed and the values for 14 response variables were calculated: sniff frequency; the number of inspiratory and expiratory sniffs; the duration, volume, average flow rate, and peak flow rate of an inspiratory and expiratory sniff; the total inspiratory and expiratory volume; and the total apneic duration. A session lasted 50 min.

An animal's sniffing behavior is a complex response pattern that varies with odorant stimuli. Although sniffing patterns can be deconstructed into a large number of descriptive variables knowledge about any single variable is insufficient to evaluate the meaning of the behavioral response to odorant stimuli (25). However, this behavior can be adequately described and evaluated by using a measure that incorporates the 14 derived variables along with their corresponding weightings (16, 18, 19, 24, 25).

Briefly, each animal contributed a 14×5 data matrix to the overall dataset, that is, 14 response variables (see above) at each of the 5 concentrations of ethanol. The 14 dimensions of the overall dataset were compressed into 2 uncorrelated dimensions (i.e., factors 1 and 2) by performing a PCA. The values (at each odorant concentration) of the 2 resultant PCA factors, therefore, defined an animal's behavioral response to the stimulus concentration. Thus,

the foregoing procedure reduced each animal's 14 × 5 data matrix to 2 response variables at each of 5 concentrations of ethanol (i.e., a 2 × 5 matrix).

To express each animal's multivariate responses as a single value that incorporates the rat's behavioral responses across all concentrations of odorant in each dimension the following procedure was used. For each PCA factor, we performed separate multivariate analyses with the 5 odorant-induced behavioral response measures as the dependent variables and maternal treatment as the independent variable. This approach provided estimates of the coefficients for each concentration of odorant in the separate regression analyses. The composite index values derived from each PCA factor for each animal was the linear summation of the constant from the regression analysis, plus the respective PCA value at each of the 5 concentrations of odorant multiplied by the appropriate estimated coefficient. This process resulted in pairs of *x* and *y* coordinates that represented the relative physical location of each ethanol-, pair-, and chow-fed animal in a behavioral response space. These data were used for analyses.

P15 Ethanol Intake. P15 rats do not drink from a bottle. Therefore, we assessed ethanol intake by infusing an ethanol solution directly into the mouth (via an intraoral cannula) and determining how much was ingested (17). After odor testing, rats were fitted with a flanged intraoral cannula (PE10 tubing; Clay Adams) (17), placed in a clean cage for 2 h and maintained with a heat lamp. By virtue of the cannula's intraoral position the pups could choose to swallow or reject the solution.

Before testing, voiding of the bowel and bladder was achieved by stimulating the ano-genital region. Pups were weighed (to the nearest 0.01 g) and placed in a plastic chamber (15 × 7 × 15 cm), and their cannula was attached to an infusion pump. After a 10-min habituation period, a 5.0% (vol/vol) ethanol solution was infused over a 15-min period with a 3-s on and 10-s off duty cycle at a rate permitting delivery of 5.5% of the animal's body weight. After testing, the animals were weighed again. Intake was calculated as grams of absolute ethanol consumed per kg of body weight.

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